Pterin interactions with distinct reductase activities of NO synthase

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Besides oxidizing L-arginine, neuronal NO synthase (NOS) NADPH-dependently reduces various electron acceptors, including cytochrome *c* and tetrazolium salts. The latter NADPH diaphorase reaction is used as a NOS-specific histochemical stain. Both reductase activities have been utilized to analyse electron transfer mechanisms within NOS. Basal L-arginine turnover by homodimeric NOS is enhanced by exogenous tetrahydrobiopterin, and the intra-subunit electron flow may include intermediate trihydrobiopterin. In the present work we have investigated the possible role of the tetrahydrobiopterin binding site of NOS in its reductase activities by examining the effects of anti-pterin type (PHS) NOS inhibitors. Although the type I anti-pterin, PHS-32, which does not affect basal dimeric NOS activity, also had no effect on either reductase activity, the type II anti-pterin, PHS-72, which inhibits basal NOS activity, inhibited both reductase activities and the NADPH diaphorase histochemical stain. Pterin-free NOS monomers catalysed both cytochrome *c* and tetrazolium salt reduction. Our data suggest that both NOS reductase activities are independent of tetrahydrobiopterin. However, occupation of an exosite near the pterin site in NOS by type II anti-pterins may interfere with the electron flow within the active centre, suggesting that steric perturbation of the pterin binding pocket or reductase interaction contribute to the mechanism of inhibition by this class of NOS inhibitors.

Key words: anti-pterins, diaphorase, nitric oxide, NO synthase mechanisms, tetrahydrobiopterin.

INTRODUCTION

Nitric oxide (NO) plays an important role both in physiology, as a signal molecule in the central nervous and cardiovascular systems [1–4], and in pathophysiology, as a cytotoxin in immune and auto-immune responses [5]. NO is generated by three genetically distinct NO synthases (NOS), neuronal (NOS-I), inducible (NOS-II) and endothelial (NOS-III) [6]. All three catalyse the conversion of L-arginine to L-citrulline and NO, NO^- (nitroxyl) or related N oxides [7–9]. However, they exhibit important differences in their modes of expression and activity regulation. NOS-I and NOS-III are constitutively expressed, whereas, in most cells, NOS-II expression requires transcriptional induction, e.g. by cytokines [6]. Enzyme activity of the constitutively expressed isoforms (NOS-I and NOS-III) is stimulated by elevated intracellular levels of free Ca^{2+} and calmodulin (CaM) [10,11], although some degree of Ca^{2+}-independent activation can be detected upon phosphorylation [12–14]. In contrast, the activity of NOS-II is independent of changes in intracellular free Ca^{2+}, as CaM is tightly bound [15].

NOS is a homodimer, and only the homodimeric conformation of NOS converts L-arginine [16]. Each monomer consists of both a reductase and a mono-oxygenase domain. The reductase domain binds NADPH and, via prosthetic FAD and FMN groups, mediates the transfer of an electron to a catalytically active haem within the oxygenase domain [17]. In addition to NADPH, FAD and FMN, the intra-subunit electron flow may include intermediate trihydrobiopterin reduction [18]. At the oxygenase domain, the physiological substrate, L-arginine, binds to the enzyme and is oxidized [19,20]. The coupling mechanism between the oxygenase and reductase domains is unclear, but is thought to occur close to the pterin binding site [18,21].

One approach to gain insight into the NOS reaction mechanism has been to dissect the overall reaction into partial reactions and to analyse each individually. These reactions include the reduction of molecular oxygen to reactive oxygen species (NADPH oxidase) and the reduction of cytochrome *c* and tetrazolium salts [e.g. Nitro Blue Tetrazolium (NBT)] [22]. The reduction of NBT (Figure 1) to a water-insoluble formazan, also termed the NADPH diaphorase reaction, is resistant to aldehyde-based protein fixatives and has been used as a histochemical stain for NOS-I in fixed tissue sections [23,24]. Enzyme kinetic analysis of the NBT reductase/NADPH diaphorase activity of NOS has been hampered by the insolubility of the resulting formazan, thus requiring the addition of organic solvents and semiquantitative assay conditions to measure product formation [23,25]. In order to assay the NADPH diaphorase activity of NOS continuously, we developed a method using the tetrazolium salt 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazolyl)-3-(4-sulphophenyl) tetrazolium (MTS). Reduction of MTS leads to a water-soluble formazan complex and thus does not require organic solvents for spectrophotometric quantification [26,27]. Cytochrome *c*, the

Abbreviations used: CaM, calmodulin; CHAPSO, 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulphonate; H_4Bip, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; MTS, 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazolyl)-3-(4-sulphophenyl) tetrazolium salt; NBT, Nitro Blue Tetrazolium; NOS, NO synthase; PHS-72 etc., anti-pterin sub-types; SOD, superoxide dismutase; TEA, triethanolamine.

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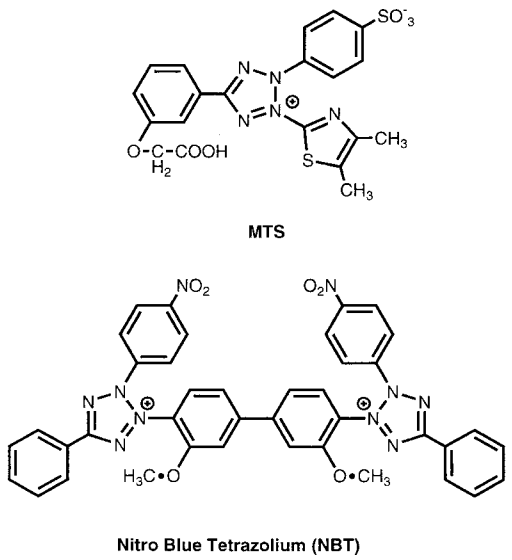


Figure 1 NADPH diaphorase substrates of NOS

Chemical structure of the tetrazolium salts MTS, generating a water-soluble formazan, and NBT, generating a water-insoluble formazan.

second reductase substrate, has been shown to bind directly to NOS-I and to be reduced in an NADPH- and Ca^{2+}/CaM-dependent manner [22].

Besides NADPH, FAD, FMN and haem, all NOS isoforms require (6*R*)-5,6,7,8-tetrahydro-L-biopterin (H_4Bip) for maximal L-arginine conversion [28,29]. The precise function of this redox-active compound in NOS is not entirely clear; it may act as a radical scavenger to stabilize the active site [30] and play a more direct role within each electron transfer cycle [18]. Since H_4Bip is not stoichiometrically consumed during L-arginine turnover, the latter hypothesis implies intramolecular pterin reduction and H_4Bip regeneration. Thus the H_4Bip binding site of NOS may be spatially linked to one of the reductase activities of the enzyme. Direct analysis of the role of H_4Bip in this interaction is perturbed by enzyme-independent, chemical interaction of H_4Bip with the redox-sensitive reductase substrates, e.g. tetrazolium salts. One recent approach to study the role of H_4Bip in L-arginine turnover and NO synthesis was the utilization of pterin-derived NOS inhibitors [30,31]. These can be divided into two major categories: type I anti-pterins (PHS), such as PHS-32 [30–32], which compete with exogenous H_4Bip, displace endogenous H_4Bip and inhibit NOS to the basal activity observed in the absence of exogenously added H_4Bip, and type II anti-pterins, such as PHS-72 [30–32], which abolish NOS activity completely and bind to NOS without displacing H_4Bip, presumably by binding to the free binding site of the partially H_4Bip-saturated enzyme. Based on molecular-field analysis of their structure–activity relationship and co-crystallization data (H. Matter, P. Kotsonis, C. S. Raman, W. Pfleiderer and H. H. H. W. Schmidt, unpublished work), this is most likely due to their binding to a hydrophobic exosite adjacent to the NOS pterin binding site [31,32]. To analyse the NOS pterin binding site and reductase interaction within NOS, we examined the effects of prototypical type I and type II anti-pterins on the reduction of cytochrome *c* and MTS by purified neuronal NOS-I, and compared these results with the possible effects of anti-pterins on the NADPH diaphorase histochemical stain.

EXPERIMENTAL

Materials

NADPH, catalase and Cu/Zn superoxide dismutase (SOD) were purchased from Roche Molecular Biochemical (Mannheim, Germany); cytochrome *c*, N^{ω}-nitro-L-arginine, 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulphonate (CHAPSO) and triethanolamine hydrochloride (TEA) were from Sigma (Deisenhofen, Germany); H_4Bip was from Dr Schircks Laboratories (Jona, Switzerland); MTS was from Promega (Madison, WI, U.S.A.); L-[2,3,4,5-^{3}H]arginine HCl (2.85 TBq·mmol^{-1}) was from Amersham (Braunschweig, Germany); the PhastGel Silver Kit, for silver staining PhastGel SDS/PAGE gels of purified NOS-I, was from Pharmacia Biotech (Freiburg, Germany). All other chemicals were of the highest purity available and obtained from either Sigma or Merck AG (Darmstadt, Germany). Water was deionized to 18 MΩ·cm in a Milli-Q apparatus (Millipore, Eschborn, Germany). Anti-pterins were synthesized as described previously [31,32].

Purification of native porcine NOS-I

Porcine cerebellum NOS-I was prepared as described previously [16,33], with slight modifications [34]. The purification protocol included ammonium sulphate precipitation of a 100000 ***g*** crude supernatant fraction followed by 2′,5′-ADP–Sepharose affinity chromatography. To obtain only the dimeric enzyme, NOS-I was further purified by gel-filtration chromatography. For this step, the eluate from the 2′,5′-ADP–Sepharose affinity chromatography (approx. 26 ml) was concentrated (Centriprep 30; Amicon, Witten, Germany) to approx. 1 ml, centrifuged at 10000 ***g*** for 10 min at 4 °C and applied to a Superose 6 HR 10/30 gel-filtration column (Pharmacia Biotech, Freiburg, Germany) operated by an FPLC system (Pharmacia Biotech). Gel filtration was performed at 4 °C at a flow rate of 0.5 ml · min^{-1} in the presence of a 20 mM TEA/HCl buffer (pH 7.5) containing 0.5 mM EDTA, 150 mM NaCl, 10 % (v/v) glycerol and 0.05 % CHAPSO. Protein was monitored spectrophotometrically at A_{280}. NOS-dimer-containing fractions were assayed for L-arginine turnover, and cytochrome *c* reductase and MTS reductase activities. The purity of NOS-I was determined by SDS/PAGE. Fractions with purity $\geqslant$ 90 % were pooled, divided into portions and stored at −80 °C before use. Pure NOS-I had a specific activity of 268.1 ± 2.9 nmol citrulline·mg^{-1}·min^{-1}.

Recombinant human NOS-I

Recombinant human NOS-I [35] was expressed in a baculovirus/Sf9 cell system [31], and purified to a specific activity of 643.0 ± 13.7 nmol of citrulline·mg^{-1} · min^{-1} by using 2′,5′-ADP–Sepharose and CaM-affinity chromatography sequentially, as described previously [16,35].

Synthesis and UV/visible absorption spectra of the MTS-derived formazan

To establish a quantitative MTS reductase assay, the enzyme-independent, direct chemical reduction of tetrazolium salts by H_4Bip was utilized. Aqueous stock solutions of MTS (5–50 μM) were converted to its formazan by adding H_4Bip in a 10-fold molar excess (50–500 μM). Subsequently, the A_{490} of different MTS concentrations was monitored against H_4Bip blanks with a UV/visible spectrophotometer (Hitachi U 2000; Hitachi, Lorch, Germany). An excellent linear correlation between the amount of MTS formazan and absorbance was found over the whole

concentration range (for three experiments, each performed in triplicate, $r^2 = 0.99$). A_{490} was therefore used to quantify NOS-catalysed MTS reduction. Calculations of total formazan were made using a molar absorption coefficient (ϵ_{490}) of $2.52 \pm 1.4 \times 10^4\ M^{-1} \cdot cm^{-1}$.

MTS reductase assay

MTS was freshly dissolved in 50 mM TEA/HCl buffer (pH 7.0). These MTS stock solutions were protected from light and kept on ice for immediate use. MTS reductase activity was assayed in 96-well microtitre plates by measuring the NOS-catalysed reduction of 2 mM MTS to its water-soluble formazan in a 100 μl reaction volume containing 0.1 mM NADPH in 50 mM TEA/HCl buffer (pH 7.0). Reactions were started by the addition of 70–80 ng of purified NOS-I. After an incubation period of 15 min at 37 °C, with protection from light, the A_{490} was measured using a ThermoMax Microplate Reader (Molecular Devices, Menlo Park, CA, U.S.A.). Blanks for each concentration of MTS were measured in the absence of enzyme and subsequently subtracted from the values obtained in the presence of NOS.

Cytochrome *c* reductase assay

Determination of cytochrome *c* reductase activity was modified from a method described previously [22]. Stock solutions of cytochrome *c* and NADPH were freshly made on the day of use. Cytochrome *c* turnover was measured spectrophotometrically by the NOS-catalysed reduction of 0.2 mM cytochrome *c* in 100 μl of 50 mM TEA/HCl buffer (pH 7.0) containing 100 μM $CaCl_2$, 100 nM CaM and 0.1 mM NADPH. Reactions were started by the addition of 70–80 ng of purified NOS and continued at 37 °C for 25 min. The total amount of reduced cytochrome *c* was calculated using a molar absorption coefficient (ϵ_{550}) of $2.1 \times 10^4\ M^{-1} \cdot cm^{-1}$.

Determination of L-arginine turnover

NOS-catalysed L-arginine turnover was determined by measuring L-[^{3}H]citrulline production from L-[^{3}H]arginine as described previously [36], with modifications [34]. Assays were performed in a reaction mixture containing 50 mM TEA/HCl buffer (pH 7.5), 20 μM L-arginine (including 5.55 kBq L-[2,3,4,5-^{3}H]arginine), 1 mM NADPH, 5 μM FAD, 10 μM FMN, 50 nM CaM, 1 mM $CaCl_2$ and 2 μM H_4Bip. Blank values were determined in the absence of enzyme. Incubations were performed at 37 °C for 15 min. The reaction was terminated by addition of stop buffer containing 20 mM sodium acetate (pH 5.5) and 2 mM EDTA at 4 °C. [^{3}H]Citrulline, and thus total L-arginine to L-citrulline conversion, was subsequently measured by liquid scintillation spectroscopy as described previously [31].

Generation and separation of NOS monomers and dimers

Under native conditions, L-arginine conversion in the absence of H_4Bip leads to NOS monomerization. NOS monomers and dimers can then be separated by size-exclusion chromatography and investigated further [30]. To generate NOS monomers, homodimeric recombinant human NOS-I (17 μg) was pre-incubated at pH 7.0 in the presence of 25 mM L-arginine, 1 mM NADPH, 5 μM FAD, 10 μM FMN, 500 nM CaM, 1 mM $CaCl_2$, 250 μM CHAPSO and 0.5 M TEA/HCl. The final incubation volume was 100 μl. Incubations were carried out at 37 °C for 15 min and stopped by the addition of 10 μl of ice-cold 5 mM EGTA. Immediately thereafter, samples were mixed, frozen in liquid nitrogen and stored at −80 °C. After thawing, samples were centrifuged at 10000 ***g*** for 10 min at 4 °C, and analysed by Superose 6 HR 10/30 size-exclusion chromatography as described previously [30,31]. NOS dimers were eluted at 12.58 ± 0.05 ml, monomers at 14.09 ± 0.06 ml, corresponding to Stokes' radii of 7.89 ± 0.01 nm and 6.07 ± 0.06 nm, which was in agreement with published values [16]. The two eluates were divided into aliquots of 300 μl, which contained purely dimeric or monomeric NOS.

NADPH diaphorase histochemical stain

Sprague–Dawley rats were anaesthetized and perfused intra-aortically with mixed aldehyde fixatives. Vibratome sections of the spinal cord, 25–50 μm thick, were incubated for 15 min in the presence of either 200 μM anti-pterin dissolved in DMSO or vehicle alone for control experiments. NADPH diaphorase staining was performed as described previously [24,37,38]. Sections were incubated at 37 °C for 1.5 h in a mixture of 0.1 % (w/v) NADPH, 0.02 % (w/v) NBT and 0.3 % (v/v) Triton X-100 in phosphate buffer (pH 7.4). Staining, using this protocol, co-localizes with immunocytochemically-defined NOS-I [24,37,38] and concentrates in lamina II–III of the superficial dorsal horn of the spinal cord [39–42].

Determination of protein

Protein concentrations were determined according to Bradford [43] using BSA as a standard.

Statistics

All results are expressed as means ± S.E.M.. Non-linear regression analysis was performed using the Prism 2.0 software package (GraphPad, San Diego, CA, U.S.A.).

RESULTS

Characterization of MTS and cytochrome *c* reductase activities

To establish MTS reductase as a novel on-line assay for the NADPH diaphorase activity of NOS, we examined the ability of native porcine NOS-I to reduce MTS. The NADPH dependency of this activity was then characterized in comparison with the established cytochrome *c* reductase and L-arginine-to-citrulline activities of NOS.

Purified porcine cerebellum NOS-I (0.6 μg), in the presence of 1 mM NADPH, was incubated with increasing concentrations of MTS (10 μM–3.2 mM), as described in the Experimental section, and the A_{490} was measured after 30 min of incubation at 37 °C. The data were fitted to a sigmoidal concentration-response curve (results of four individual experiments performed in triplicate; $r^2 = 0.99$), which indicated the saturable, enzymic formation of formazan. NOS-I thus reduced MTS to a water-soluble formazan complex in a concentration-dependent manner with a K_m[MTS] value of 600.8 ± 1.1 μM and a specific activity (V_{max}) of up to 1.8 μmol $mg^{-1} \cdot min^{-1}$.

Thereafter, NOS-I was assayed for MTS (50 ng of NOS-I) and cytochrome *c* (75 ng of NOS-I) reductase activities. Reductase activities, at 37 °C, were monitored over 30 min at 1 min intervals and total amounts of formazan and reduced cytochrome *c* were calculated. Linear regression analysis was performed for three experiments, each performed in triplicate. MTS reductase activity remained stable over a period of 15 min, and declined thereafter; cytochrome *c* turnover was linear for at least 30 min. The kinetics of MTS turnover thus resembled those of L-arginine turnover, which is also linear for approx. 15 min under similar conditions.

Table 1 Comparison of distinct NOS activities

L-Citrulline formation, MTS reduction and cytochrome *c* reduction by purified porcine cerebellum NOS-I were determined, as described in the Experimental section, in the presence of various concentrations of NADPH and CaM. Subsequently, non-linear regression analysis was performed to calculate K_a or K_m values. All activities were NADPH-dependent; however, only cytochrome *c* and citrulline formation were enhanced by the addition of Ca^{2+}/CaM.

	K_m NADPH (μM)	EC_{50} CaM (nM)	V_{max} (nmol · min^{-1} · mg^{-1})
Citrulline formation	10.3 ± 3.2	5.3 ± 1.5	268 ± 3
MTS reduction	20.9 ± 1.7	No dependence	1370 ± 170
Cytochrome *c* reduction	9.9 ± 6.4	14.1 ± 1.2	5489 ± 213

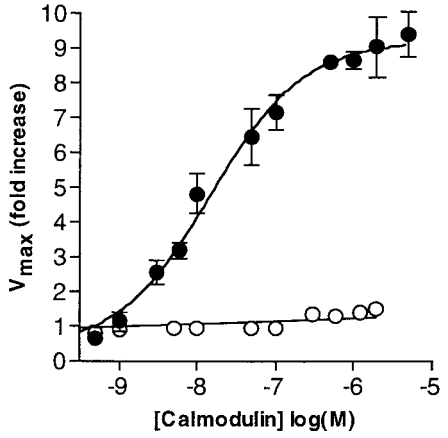


Figure 2 Effects of CaM on cytochrome *c* and MTS reductase activities of NOS-I

Purified porcine cerebellum NOS-I (140 ng) was incubated in the absence or presence of CaM (0.1 nM–2 μM) with either cytochrome *c* (●) or MTS (○). All other assay conditions were the same as described in the Experimental section. Control values were determined in the absence of CaM. Each value represents the mean ± S.E.M. of three individual experiments, each performed in triplicate. The effects of increasing concentrations of Ca^{2+}/CaM on cytochrome *c* reductase activity were analysed by fitting a sigmoidal concentration response curve to the data points ($r^2 = 0.99$). Cytochrome *c* reduction by NOS-I was found to be greatly enhanced by Ca^{2+}/CaM in a concentration-dependent manner, whereas the formation of MTS-derived formazan was not significantly affected.

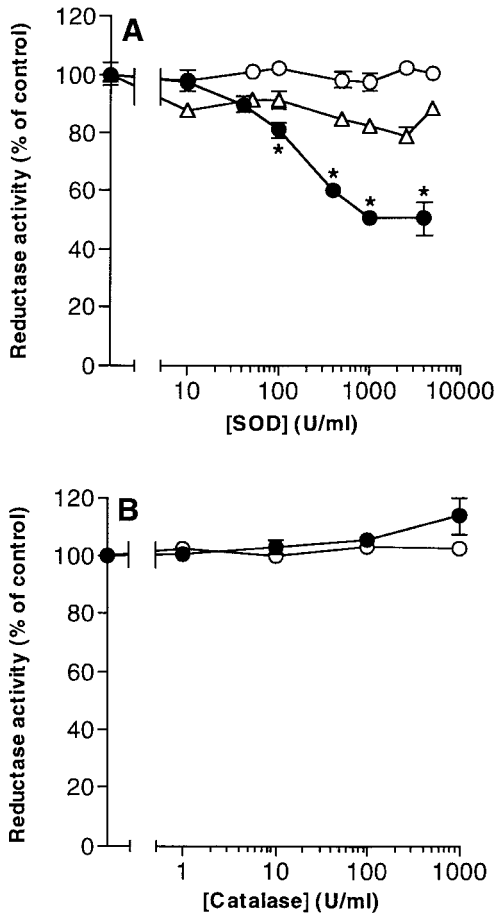


Figure 3 Effect of SOD and catalase on cytochrome *c* and MTS reductase activity of NOS-I

(**A**) Cytochrome *c* (○, in the presence of 100 μM $CaCl_2$ and 100 nM CaM; ●, in the absence of Ca^{2+}/CaM) and MTS (△) reductase activities were determined in the presence of purified porcine cerebellum NOS-I (115 ng) and increasing concentrations of SOD [10–5000 units (U) · ml^{-1}]. MTS reduction was found to be significantly inhibited by SOD, whereas cytochrome *c* turnover was affected neither in the presence nor in the absence of Ca^{2+}/CaM. (**B**) Cytochrome *c* (○, in the presence of 100 μM $CaCl_2$ and 100 nM CaM) and MTS reductase (●) activities were determined as above, but in the presence of increasing concentrations of catalase (0.1–1000 units · ml^{-1}) instead of SOD. No effect of catalase was observed on cytochrome *c* or on MTS reduction, arguing against a role for H_2O_2 in the reductase activities of NOS-I. All other assay conditions were the same as described in the Experimental section. Activity is expressed as a percentage of the respective control, e.g. in the presence of Ca^{2+}/CaM, and in the absence of SOD or catalase. Data points represent means ± S.E.M. of three individual experiments, each performed in triplicate. The asterisk (*) indicates significant difference from the respective control, $P < 0.01$.

To further characterize the MTS reductase assay, we determined the apparent K_m value for NADPH and compared it with the respective value in the cytochrome *c* assay. NOS-I (140 ng) was incubated with MTS or cytochrome *c*, as described in the Experimental section, with increasing concentrations of NADPH (100 nM–10 mM). Both MTS and cytochrome *c* reductase activities were NADPH-dependent with K_m[NADPH] values in the same range as for L-arginine turnover (Table 1). Non-linear regression analysis was performed and resulted in a sigmoidal concentration response curve for both MTS ($r^2 = 0.98$) and cytochrome *c* turnover ($r^2 = 0.98$).

Ca^{2+}/CaM was without effect on MTS turnover, but stimulated cytochrome *c* reduction

MTS reductase activity was independent of CaM in the range of 0.1 nM to 2 μM, in the presence of saturating Ca^{2+} concentrations (Figure 2). Conversely, cytochrome *c* reductase activity was largely dependent on Ca^{2+}/CaM (Figure 2), which stimulated basal cytochrome *c* reduction approx. 9-fold. The half-maximally effective concentration (EC_{50}) of CaM for stimulating cytochrome *c* reduction by NOS was similar to its EC_{50} value for stimulating citrulline formation (Table 1).

Effects of SOD on MTS and cytochrome *c* reductase activities

The extend to which NOS reductase activities are mediated by intermediate superoxide (O_2^-) formation is controversial [44,45]. To address this issue, the effects of SOD on MTS and cytochrome *c* reductase activities of native porcine NOS-I were investigated. SOD inhibited MTS reduction at concentrations as low as 100 units · ml^{-1}. At 1000 units · ml^{-1}, inhibition by SOD was maximal (50 % of control). In contrast, even the highest concentration of SOD tested (5 k-units · ml^{-1}) had no effect on the Ca^{2+}/CaM-stimulated reduction of cytochrome *c* (Figure 3A).

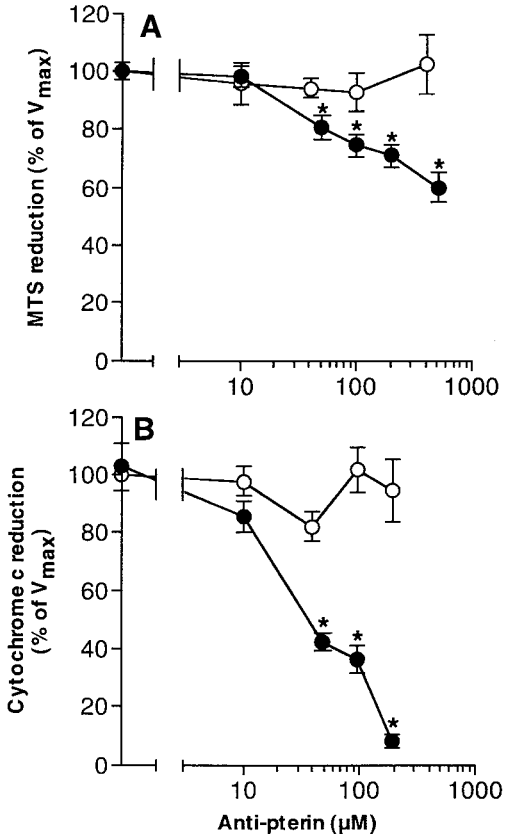


Figure 4 Effect of type I and II anti-pterins on MTS and cytochrome *c* reductase activities of purified porcine NOS-I

(**A**) Purified porcine cerebellum NOS-I (70 ng) was incubated in the presence of MTS (2 mM), NADPH (0.1 mM), and various concentrations of PHS-32 (○) or PHS-72 (●). The type II anti-pterin PHS-72 significantly inhibited MTS reductase activity, whereas the type I anti-pterin PHS-32 had no effect. Control values were determined in the absence of anti-pterins and in the presence of NOS and solvent (10% DMSO). The activity of the samples is expressed as a percentage of the respective control. Data points represent means ± S.E.M. of three individual experiments, each performed in triplicate. (**B**) Purified porcine cerebellum NOS-I (70 ng) was incubated in the presence of cytochrome *c* (0.2 mM), NADPH (0.1 mM), $CaCl_2$ (100 μM), CaM (100 nM) and various concentrations of PHS-32 (○) or PHS-72 (●). The anti-pterin II PHS-72 significantly inhibited cytochrome *c* reductase activity of NOS-I, whereas the anti-pterin PHS-32 had no effect. Except for the addition of DMSO and anti-pterins, assay conditions were as described in the Experimental section. Controls contained enzyme and solvent (10% DMSO), but no anti-pterins. Activity is reported as a percentage of V_{max}. Data points represent means ± S.E.M. of three experiments, each performed in triplicate. The asterisk (*) indicates significant difference from control, $P < 0.001$.

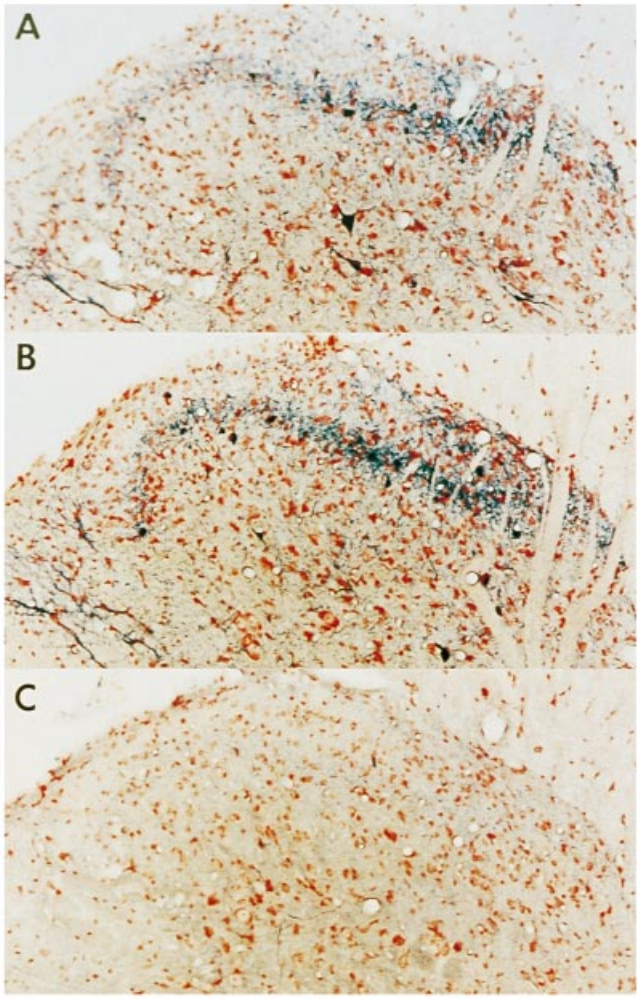


Figure 5 Effect of anti-pterins on NADPH diaphorase histochemical stain

Vibratome sections of rat spinal cord, known to contain high levels of neuronal NO synthase (NOS-I), were processed for NADPH diaphorase activity, as described in the Experimental section, after incubation with 200 μM anti-pterin or vehicle. Control staining (**A**) without anti-pterin was concentrated in laminae II–III of the dorsal horn. Preincubation of sections with 200 μM PHS-32 (**B**) showed the same staining pattern as the control, whereas pre-treatment with 200 μM PHS-72 (**C**) markedly inhibited NADPH diaphorase staining. Data are representative of three independent experiments.

Since a portion of cytochrome *c* reduction is CaM-independent, we also characterized this basal activity. However, up to 5 k-units · ml^{-1} SOD had no effect on cytochrome *c* reduction, regardless of whether Ca^{2+}/CaM was present or not, suggesting that neither basal nor CaM-stimulated reduction of cytochrome *c* is mediated by O_2^- (Figure 3A).

Catalase affects neither MTS nor cytochrome *c* reduction

Instead of intermediate O_2^-, NOS may also form H_2O_2 (A. Reif, Z. Shutenko, M. Feelisch and H. H. H. W. Schmidt, unpublished work), which may interfere with MTS or cytochrome *c* reduction. Therefore the effects of various concentrations of catalase (0.1–1000 units · ml^{-1}) on MTS and cytochrome *c* reductase activities were examined. However, catalase affected neither MTS nor cytochrome *c* reduction (Figure 3B). Thus the lack of effect of SOD on the cytochrome *c* reductase of NOS cannot be explained by intermediate H_2O_2 formation.

Effects of anti-pterins on MTS and cytochrome *c* reduction

To investigate the possible role of NOS-associated H_4Bip or the NOS pterin binding site for the two NOS reductase activities studied, we examined the effect of one prototypic compound for each of the two classes of anti-pterin on MTS and cytochrome *c* reduction. Figure 4(A) shows the effect of type I (PHS-32) and type II (PHS-72) anti-pterins on MTS reductase activity of native NOS-I, expressed as a percentage of the control, i.e. without added anti-pterin. PHS-32 had no effect on MTS turnover in concentrations of up to 400 μM. Conversely, PHS-72 induced a significant, concentration-dependent inhibition of formazan formation down to 60% of control.

Table 2 Expression of MTS and cytochrome *c* reductase activity by both pterin-containing dimers and pterin-free monomers

Recombinant human NOS-I (17 μg) was separated into pterin-containing dimers and pterin-free monomers by size-exclusion chromatography as described in the Experimental section. Subsequently, 40 μl (L-arginine conversion, cytochrome *c* reduction) and 50 μl (MTS reduction) of the same dimer and monomer peak fractions were assayed for citrulline formation, MTS and cytochrome *c* reductase activities as described in the Experimental section. The values represent absolute amounts of product formed · min^{-1} · ml^{-1}; n.d., not detectable.

	Monomeric NOS-I	Dimeric NOS-I
Citrulline formation (pmol)	n.d.	19.3 ± 0.1
MTS reduction (pmol)	1850 ± 270	2000 ± 130
Cytochrome *c* reduction (pmol)	8620 ± 100	8580 ± 300

The effects of the same anti-pterins on cytochrome *c* reductase activity of native porcine NOS-I are shown in Figure 4. Similarly to MTS reduction, the type I PHS-32, in concentrations of up to 200 μM, had no significant effect on cytochrome *c* reduction. In contrast, the type II PHS-72 significantly decreased cytochrome *c* turnover in a concentration-dependent manner. At 200 μM, this compound diminished cytochrome *c* reduction to 8 % of the control value. These data suggest a selective inhibitory effect of type II anti-pterins, which occupy not only the NOS pterin binding site in a H_4Bip-competitive manner, but also an adjacent, hydrophobic exosite. The latter is not competitive with H_4Bip, and may interfere sterically with subdomain interactions, e.g. oxygenase/reductase coupling.

The effects of anti-pterins are preserved in the NADPH diaphorase histochemical stain

An assay *in vitro* for NADPH diaphorase activity of neuronal NOS using MTS can only be an approximation of the reactions which occur during the NADPH diaphorase histochemical staining procedure using NBT. Therefore we investigated the effects of the different anti-pterins on NADPH diaphorase stains with NBT in neuronal tissue sections (Figure 5). Staining in the absence of anti-pterins (Figure 5A) was concentrated in lamina II–III of the superficial dorsal horn. In the presence of 200 μM of type I PHS-32 (Figure 5B), the NADPH diaphorase stain remained unaffected, showing the same staining pattern as the control sections (Figure 5A). However, 200 μM of the type II PHS-72 (Figure 5C) almost abolished NADPH diaphorase activity staining in the tissue sections. These findings are consistent with the data derived from the MTS assay *in vitro*, and strengthen the hypothesis that MTS is a substrate for the NADPH diaphorase reaction and that this activity is selectively altered by the type II PHS-72.

Both pterin-containing dimers and pterin-free monomers of NOS-I have MTS and cytochrome *c* reductase activity

H_4Bip is also involved in NOS dimer stabilization [30], but present data do not allow differentiation of the possible roles for H_4Bip in MTS and cytochrome *c* reductase activities in dimeric and monomeric NOS-I. Thus recombinant human NOS-I was separated into pterin-containing dimers (6.13 ± 0.39 pmol H_4Bip per fraction) and pterin-free monomers. Both states of NOS, dimeric and monomeric, occur during NOS-catalysed L-arginine turnover [30] or can be generated in the presence of various protein denaturants, such as SDS [46] or urea [47]. However, both NOS-I dimers and monomers were competent for MTS and cytochrome *c* turnover (Table 2), suggesting that these NOS activities are pterin-independent but are affected by pterin exosite occupation by the type II PHS-72.

As we observed cytochrome *c* turnover by monomeric NOS, we investigated whether NOS monomers also required Ca^{2+}/CaM to reduce cytochrome c. We therefore incubated monomeric NOS, as described above, with and without $CaCl_2$ (100 μM) and CaM (100 nM). In the absence of both Ca^{2+} and CaM, only 24.89 ± 2.42 % of the V_{max}, i.e. the activity in the presence of Ca^{2+} and CaM, was observed in three experiments, each performed in triplicate. The electron flow to the haem in monomeric NOS therefore also appears to be regulated by CaM.

DISCUSSION

In addition to L-arginine oxidation, NOS NADPH-dependently reduces artificial electron acceptors such as tetrazolium salts [23], cytochrome *c* [22] and others [45]. The reduction of the tetrazolium salt NBT to a water-insoluble formazan complex, termed the NADPH diaphorase reaction, is widely used as a selective histochemical marker for NOS-I in aldehyde-fixed sections of neuronal and other tissues [23,48,49]. Yet it is mechanistically not understood which domain in NOS mediates this reaction.

In the present work, we demonstrate that purified porcine cerebellar NOS also reduces the tetrazolium salt MTS. Using MTS as an electron acceptor to quantify NADPH diaphorase activity has the advantage of forming a water-soluble formazan complex, which allows enzyme kinetic analysis. NOS-dependent formation of MTS formazan mirrored the kinetics of L-arginine to L-citrulline conversion [34]. Similarly to the reduction of NBT, MTS turnover showed an absolute requirement for NADPH. As both substrates are tetrazolium salts, MTS can be considered as an alternative substrate for the NADPH diaphorase activity of NOS-I. This is corroborated by the qualitatively similar inhibition pattern of anti-pterins on all three reductase activities, cytochrome *c* reductase MTS reductase and NADPH diaphorase.

Calmodulin triggers the flavin-to-haem electron transfer between the reductase and oxygenase domains of the enzyme [50], increases the rate of electron transfer into the flavins [51], and is therefore essential for maximal L-arginine-to-L-citrulline conversion. The reduction of cytochrome *c*, but not that of MTS, was enhanced by CaM in a concentration-dependent manner. This profound difference in Ca^{2+}/CaM-mediated catalysis of MTS and cytochrome *c* emphasizes the important and specific role of CaM binding between the haem and flavin-binding domains of all NOSs. Calmodulin presumably affects the orientation of these domains, thereby modulating and de-inhibiting [45] electronic coupling of the reductase-to-oxygenase domain.

In contrast, the effects of Ca^{2+}/CaM on the NADPH diaphorase activity of NOS remain unclear. NBT formazan formation is generally believed to be Ca^{2+}/CaM-independent, although one report found it to be increased by Ca^{2+}/CaM [44]. In our hands, MTS reduction was independent of Ca^{2+}/CaM, consistent with most other reports [25]. The possibility exists that enzyme-bound H_4Bip is oxidized by MTS (see the Experimental section), thus influencing the CaM sensitivity of the enzyme. However, this would imply an interference in the H_4Bip and the CaM binding sites, which has not been observed experimentally [52]. Furthermore, NOS is only partially saturated with endogenously bound H_4Bip [30,31], so that changes in the H_4Bip redox state should only affect a sub-fraction of purified enzyme. Thus H_4Bip oxidation is unlikely to account for the observed differences in the CaM dependence between MTS and cytochrome *c* reductase activities.

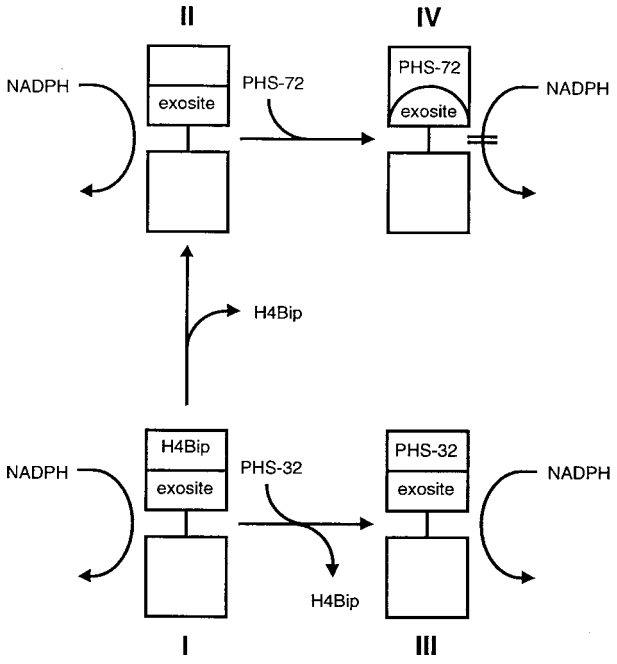
It has been shown that Ca^{2+}/CaM increases cytochrome *c* reduction by NOS up to 10–15 fold, whereas it stimulates the rate

of reduction of the artificial electron acceptors 2,6-dichlorophenolindophenol and ferricyanide only slightly or not at all [45]. This is in good agreement with our present data on both the CaM-dependence of cytochrome *c* reduction and the CaM-independence of MTS reduction. Since the formation of L-citrulline from L-arginine is CaM-dependent, cytochrome *c* reduction appears to follow a similar mode of electron transfer, despite the fact that direct protein–protein interaction occurs [22]. Moreover, a variety of electron acceptors (natural and artificial) interfere with NO formation, because of the low substrate specificity of the reductase domain of NOS [22]. Presumably, due to competition with molecular oxygen for NADPH-derived electrons, these acceptor molecules inhibit L-citrulline formation in a concentration-dependent manner [23,44].

The role of intermediate superoxide for the cytochrome *c* reductase activity of NOS-I, in addition to direct protein–protein interaction, remains a matter of debate [44,45]. Ca^{2+}/CaM stimulates the NADPH-dependent reduction of cytochrome *c* by NOS in a SOD-sensitive manner (500 units of SOD $\cdot$ ml^{-1}), suggesting that cytochrome *c* reduction is mediated by intermediate superoxide [45]. However, others have found that NOS directly binds and reduces cytochrome *c* in an SOD-insensitive manner [22,44] when using up to 1000 units of SOD $\cdot$ ml^{-1}. In the present study, both the basal (Ca^{2+}/CaM-independent) and the CaM-dependent cytochrome *c* reductase activities were SOD- as well as catalase-insensitive, arguing against a role for intermediate superoxide (and H_2O_2), similar to the NADPH-dependent cytochrome P450 reductase [22,53,54]. Furthermore, the lack of effect of SOD on basal cytochrome *c* turnover indicates that the mechanisms of cytochrome *c* reduction are similar in the presence and absence of Ca^{2+}/CaM.

Conversely, the MTS reductase activity of NOS was, at least in part, mediated by O_2^- but not by H_2O_2. Based on our results, approximately half of the NOS-catalysed MTS reduction was mediated by direct transfer of electrons to MTS, fulfilling the definition of an NADPH diaphorase reaction, whereas the other half was reduced by NOS-derived superoxide anions. In the case of the NADPH diaphorase histochemical stain, the latter reaction might be excluded because of the presence of SOD in neuronal cells, thus resulting in the apparently uniform behaviour of this reaction *in situ*. This may also explain why PHS-72 is a more effective inhibitor of the NADPH diaphorase histochemical reaction when compared with the MTS reductase activity of purified NOS.

Recently, the concept of a stoichiometric redox role for H_4Bip [18,55] in catalytically active NOS has been put forward. The intermediate formation of H_3Bip$^\bullet$, yet with almost full recovery of H_4Bip, requires that H_4Bip is recycled by an intrinsic reductase activity. This reductase activity should occur independently of Ca^{2+}/CaM (and at higher rates than H_4Bip consumption), and thus be different from Ca^{2+}/CaM-dependent oxygen activation by NOS. In order to clarify the role of enzyme-associated pterin or the NOS pterin binding site *per se* in the two reductase activities of NOS investigated, the effects of recently developed anti-pterins [31,32] on cytochrome *c* and MTS reductase activities were tested (see Figure 4). PHS-32, a type I anti-pterin, which displaces $\geqslant 80\,\%$ of the endogenously bound H_4Bip [30,31] without being a partial agonist itself, was without effect on MTS or on cytochrome *c* turnover. This suggested that H_4Bip neither has a direct role in the electron transfer leading to the reduction of MTS and cytochrome *c* by native NOS-I nor does it compete with these substrates. However, a recent study detected an enzyme-bound H_3Bip$^\bullet$-radical by EPR analysis [55]. A unifying hypothesis of both this and our findings [8] suggests that H_4Bip is not necessarily involved in the electron flow and L-arginine turnover of the enzyme but helps in coupling NADPH oxidation to product formation (NO and H_2O_2 instead of NO^- and O_2^-, in the presence or absence of L-arginine respectively), resulting in the transient formation of a pterin radical.



Scheme 1 Schematic representation of the effects of H_4Bip and anti-pterins on the reductase activities of NOS

H_4Bip-containing NOS monomers and dimers reduce cytochrome *c*, MTS and NBT by using NADPH as a source for electrons (I). These reductase activities remain intact after removing H_4Bip from the enzyme (II). The type I anti-pterin, PHS-32, replaces endogenously bound H_4Bip from its binding site, but preserves the reductase activities of the enzyme (III). PHS-72, a type II anti-pterin, is unable to displace endogenously bound H_4Bip but binds to unoccupied pterin binding sites and the adjacent exosite of NOS. The latter is suggested to disrupt the electron flow of the enzyme, which is required for both reductase activities of NOS (IV).

PHS-72, a type II anti-pterin, does not readily displace endogenous H_4Bip. In analogy to NOS-I-catalysed L-arginine turnover, the inhibitory effect of PHS-72 seems to originate from possible conformational changes of the enzyme and/or blockade of an exosite by the bulky substituents at the C-6 and C-7 position of type II anti-pterins [31,32]. This structure–activity relationship was recently confirmed by comparative molecular-field analysis (H. Matter, W. Pfleiderer and H. H. H. W. Schmidt, unpublished work). Thus type II anti-pterins may disrupt electron flow from within the pterin binding pocket of the enzyme, without a need to interfere directly with endogenous H_4Bip (Scheme 1). This might also explain the stronger effect of PHS-72 on cytochrome *c* versus MTS reduction. However, NOS reductase activities do not depend on protein-associated pterin, as both pterin-free monomers and pterin-containing dimers of recombinant human NOS-I expressed similar MTS and cytochrome *c* reductase activities (see Table 2). Monomeric NOS may re-assemble to form dimeric enzyme, which is only functional for MTS/cytochrome *c*, but not for L-arginine conversion. However, this seems unlikely as neither L-arginine nor H_4Bip were present in our incubation mixtures. In previous studies in our laboratory [30] and in other laboratories [47,56–58], re-dimerization of NOS monomers was not found in the absence of both L-arginine and H_4Bip. It is thus generally accepted that

monomeric neuronal NOS absolutely depends on the presence of L-arginine to form the dimeric enzyme. Reduction of MTS (Ca^{2+}/CaM-independently) and of cytochrome *c* (Ca^{2+}/CaM-dependently) by monomeric NOS-I is in agreement with a previous study [58] reporting cytochrome *c* reductase activity of monomeric NOS-II. Most interestingly, cytochrome *c* reductase activity in monomeric NOS was also dependent on the presence of CaM. The CaM-binding site therefore seems to be intact in the NOS monomer, and electron flow can occur between the reductase and the oxygenase domains upon Ca^{2+}/CaM binding.

We thus conclude that NOS displays distinct reductase activities with respect to their Ca^{2+}/CaM dependence, SOD and anti-pterin sensitivity. In none of them does NOS-associated H_4Bip play a role. However, occupation of the NOS pterin binding site and adjacent exosite by the type-II PHS-72 interferes with the intrasubunit electron flux, possibly by affecting oxygenase/reductase coupling. With the resolution of the structure of the oxygenase domains of two NOS isoforms [18,21], anti-pterins will be a valuable tool to analyse further the Ca^{2+}/CaM-dependent coupling of this domain to the reductase site and intermediate $H_3Bip^{\bullet}$ formation.

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